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Targeting Immunological "Restrainers": Understanding the Immunology behind Combination Chemoimmunotherapy to Improve the Treatment of Malignant Mesothelioma

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14. ABSTRACT

This study looks at the role the body's immune system plays during mesothelioma tumour development with a specific focus on a subset of immune cells called Treg that act to limit anti-tumour immunity. Our preliminary studies indicated that a number or previously published inhibitors of Treg cells are not effective in our models. We employed a new animal model which allows for the depletion of Treg cells in a very controlled manner and observed that targeted removal of Treg, particularly during early tumour development can significantly enhance anti-tumour immunity and delay tumour development. We are currently investigating whether Treg removal in combination with chemotherapy can improve survival outcomes relative to individual therapies. Additionally, we have observed that asbestos induced mesothelioma development is slower in mice that lack a functional immune system compared to mice that are immune competent. Experiments are currently underway to establish the immunological mechanisms behind these interesting findings. On completion of this study we will have a greater understanding of the role host immunity plays during early stages of tumour development and subsequent treatment of established tumours and will use this information to develop improved therapies for the treatment of malignant mesothelioma.

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Mesothelioma, immunotherapy, chemotherapy. Combination therapy.

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Targeting immunological "restrainers": Understanding the immunology behind combination chemo-immunotherapy to improve the treatment of MM.

Introduction: Malignant mesothelioma (MM) is a highly aggressive, incurable asbestos-induced cancer that is increasing in incidence globally. Treatment for mesothelioma is predominantly palliative, with median survival for patients undergoing chemotherapy around 12 months. This poor prognosis highlights the need for improved treatment modalities. A promising new approach for treating cancer has been to combine chemotherapy with immunotherapy. Chemo-immunotherapy has shown survival benefits over chemotherapy alone in the treatment of metastatic melanoma. However, there are few studies that have investigated whether immunotherapy may enhance the outcome of standard first line chemotherapy treatment for MM.

Our program has focussed on understanding the immunobiology associated with tumour development. Using two unique animal models of mesothelioma, we have demonstrated that the combination of immunostimulatory chemotherapy with an immunotherapy that drives a strong anti-tumour immune response effectively targets and destroys tumour cells. Despite this, not all animals are cured and our data suggests that this is most likely due to the presence of immunological processes that "restrain" an otherwise effective anti-tumour immune response. Regulatory T cells (Tregs) have been identified as playing a critical role in suppressing anti-tumour immunity. While a variety of agents that specifically target Tregs have recently been identified, the role of Tregs in the development of MM has yet to be properly investigated. ***The purpose of the work described in this grant is to evaluate the efficacy of Treg-specific immunotherapies in combination with immunostimulatory chemotherapy to improve the treatment of MM.*** The preliminary data generated by this pre-clinical work will inform and facilitate the development of new clinical trials in mesothelioma.

This report covers the worked completed to date with respect to Aims 1a, 1b and 1c listed below. We have also included a brief summary, regarding ongoing and future work related to Aims 2a, 2b and 2c.

Body: Objectives: Specific Hypotheses and Aims: The adaptive immune response plays a key role in the early changes associated with mesothelial cell transformation and tumour development, but is inhibited by immunological "restrainers". Ablation of these restrainers will enhance anti-MM immunosurveillance (during tumour development) and improve the therapeutic efficacy of current treatment regimens for established MM disease.

Aim1. Ablation of restrainers of the anti-tumour immune response, particularly regulatory T cells, will enhance the therapeutic efficacy of chemotherapy. (A) To identify in vivo which Treg-specific immunotherapy (P60, CCR4 antagonist or low dose CY) is best suited for combination chemoimmunotherapy with gemcitabine, pemetrexed, or cisplatin. (B) Determine optimal dose / schedule for best combination treatment identified in aim 1a. (C) Assess optimised treatment parameters in the clinically relevant MexTAg mouse model.

Aim 1a: Identify which Treg-specific immunotherapy (P60, CCR4 antagonist or LDCY) is best suited for combination chemoimmunotherapy with gemcitabine, pemetrexed, or cisplatin.

Preliminary experiments were set up to establish which chemotherapy would be suitable to use in combination with Treg inhibitors. Initial experiments (Figure 1) indicated that gemcitabine (gem) significantly delayed tumour growth compared to untreated control mice ($p < 0.001$) with 40% of gem treated animals showing complete tumour regression (panels A & B). In contrast to gemcitabine, there was no significant difference observed for AB1-HA tumour growth when mice were treated with cisplatin or pemetrexed (panels 1 C through F).

Based on these results we decided not to pursue any further experiments using pemetrexed or cisplatin and focused on assessing Treg depletion in combination with gemcitabine.

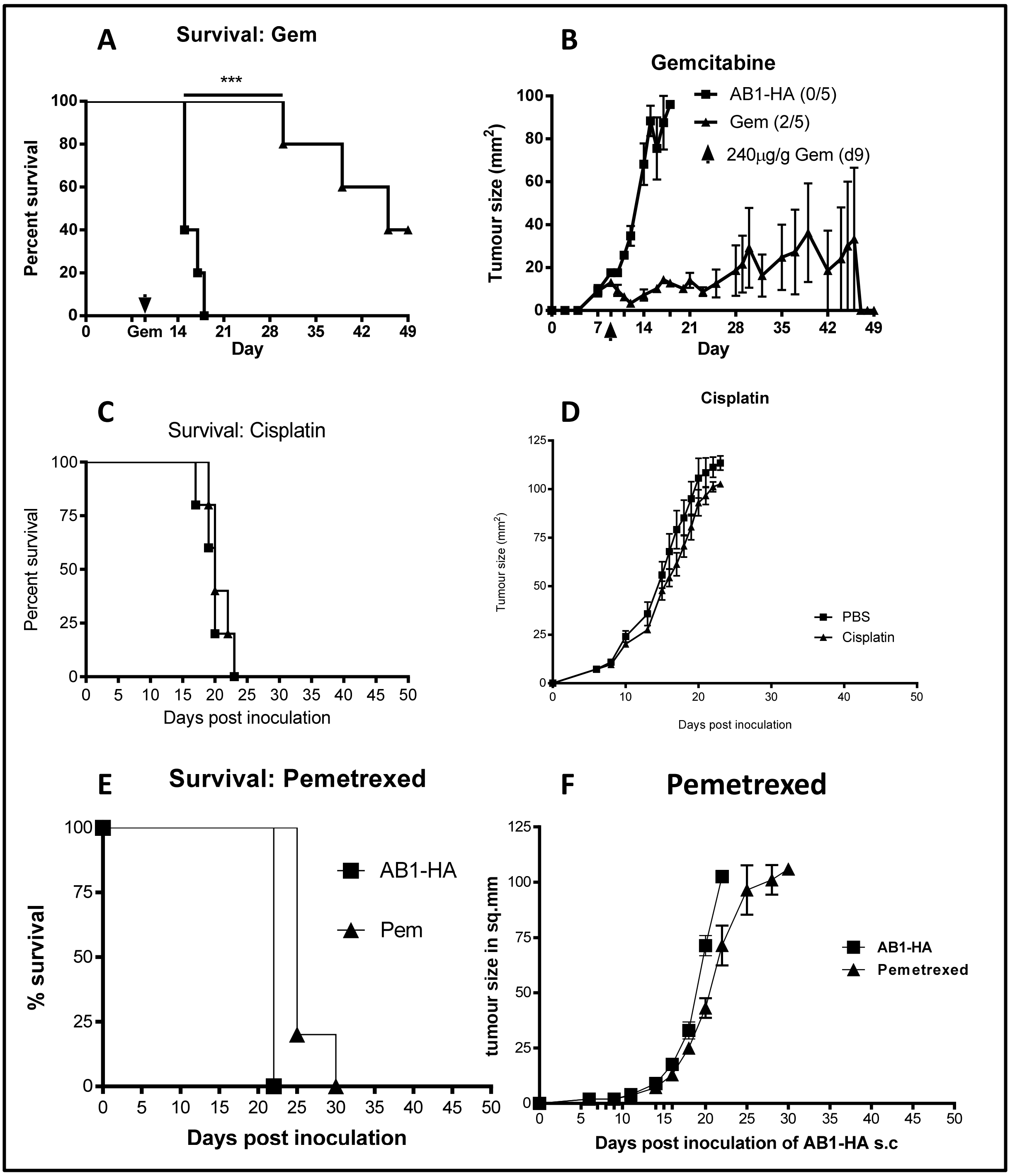

Figure 1: Survival and tumour growth curves depicting the effect of individual chemotherapy on AB1-HA tumour bearing BALB/c mice. (A-B) Gemcitabine: 240 µg/g/mouse d9; (C-D) Cisplatin: 6 µg/g/mouse d9 and (E-F) Pemetrexed: (60 µg/g/mouse d7/8/9 & d14/15/16).

Assessing the role of Treg inhibitors in AB1-HA tumour bearing mice: Before assessing the effect of combining the different Treg inhibitors, P60, CCR4 antagonist AF399 and low dose cyclophosphamide with gemcitabine, we first needed to establish whether these reagents would work in our model at the published dose. Groups of AB1-HA mice were inoculated with subcutaneously $5x10^5$ AB1-HA cells and left untreated or treated with individual Treg inhibitors and overall survival and tumour growth monitored. Mice were culled when tumour reached 100 mm^2 as per UWA animal ethics approvals.

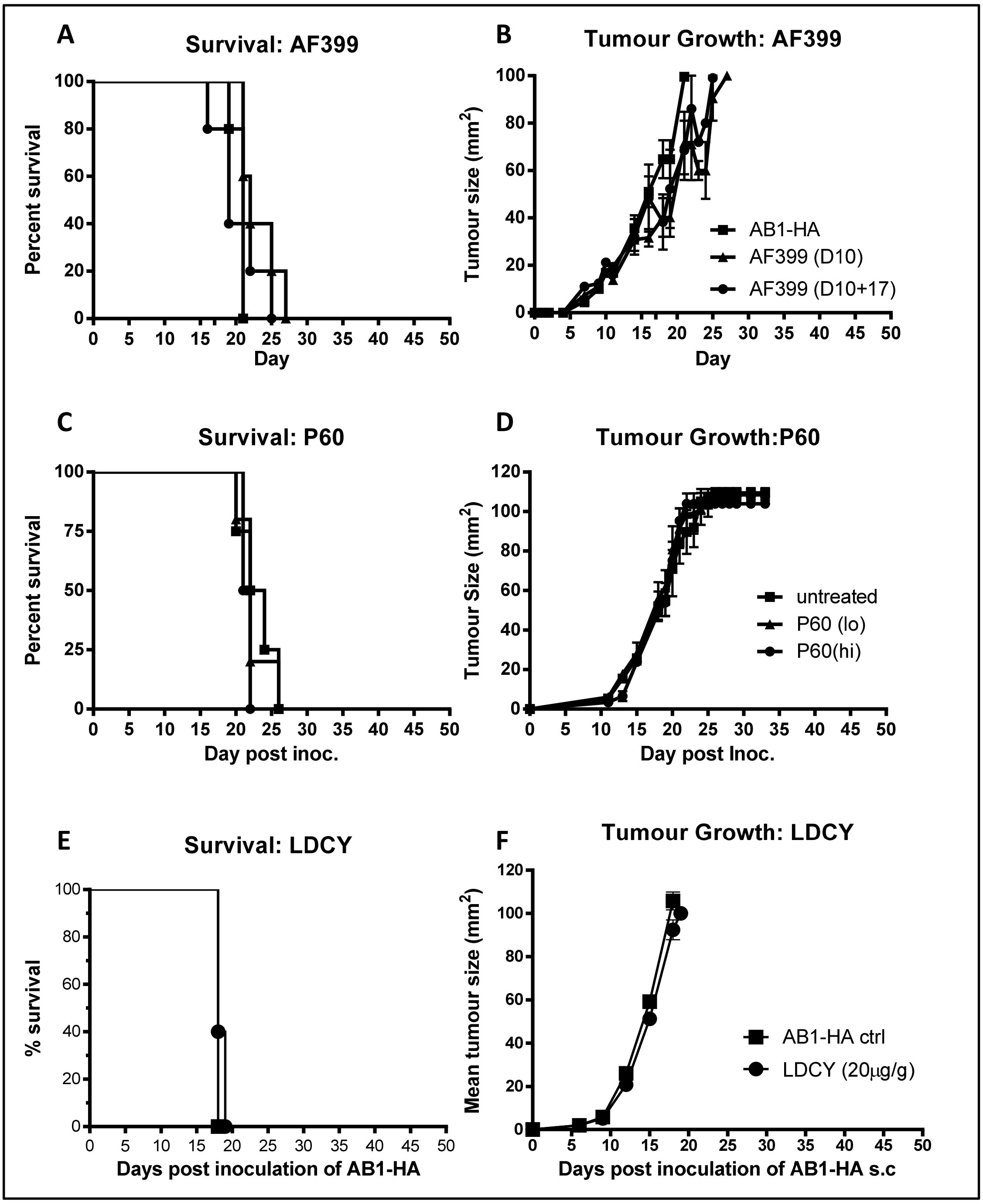

Figure 2: Survival and tumour growth of Ab1-HA bearing BALB/c mice following individual Treg inhibitor treatment. (A-B) CCR4 antagonist AF399 (3 μg/g i.p. on indicated days). (C-D) Small molecule inhibitor P60 low dose (50 μg/dose, q1dx10) and a high dose (100 μg/dose, q3dx3) staring day 9. (E-F) low dose cyclophosphamide (20 μg/g, q3dx5).

We were surprised to see that there was no significant delay in tumour growth between untreated controls and mice receiving any of the three Treg inhibitors as individual monotherapies. This is despite treatments being administered at equivalent, or higher than, the published doses for these therapies. From these results we concluded that the P60 small molecule inhibitor of FoxP3 and the CCR4 antagonist were unlikely to be effective in our AB1-HA animal model. Although there may be scope to increase the dose of cyclophosphamide, additional work in our laboratory has indicated that doses between 100-200 μg/dose can result in complete tumour regression and therefore care must be taken when using higher doses of cyclophosphamide in combination with gemcitabine.

Targeted depletion of regulatory T cells using FoxP3.dtr.crls transgenic mice.

The above experiments indicated that it would be unlikely that we could investigate the role of regulatory T cells in tumour development using the reagents we had proposed. To overcome this problem, we decided to make use of the BALB/c FoxP3.dtr.crls transgenic mouse model, which was not available to us at the time of the grant application. The BALB/c FoxP3.dtr.crls mice (referred to as FoxP3.dtr mice herein) have been genetically modified such that the gene encoding the receptor for Diphtheria Toxin (DTR) has been cloned under the control of the FoxP3 promoter, which results in expression of the DTR on FoxP3 expressing cells, such as Treg. This model enables the specific depletion of FoxP3 expressing cells after the administration of Diphtheria toxin (DTX), without any detrimental effect to other immune cell populations, such as CD4 and CD8 T cells, or to the mouse in general. These experiments did not commenced in January 2013, after approval was granted for the inclusion and use of FoxP3.dtr mice by both the UWA and US Dept. Defence ACURO animal ethics committees.

We first sought to assess the effect of DTX administration on Tregs by administering different doses of DTX to FoxP3.dtr mice. Groups of naive FoxP3.dtr were administered of DTX as sequential intra peritoneal injections on two consecutive days. As shown in Figure 3, DTX administration resulted in a dose depended reduction in CD4+ FoxP3+ Treg (diamonds) without affecting other lymphocytes subsets or the levels of Tregs from normal BALB/c mice. However, DTX mediated Treg depletion was only transient, with the proportion of Treg rebounding four days after DTX administration. Interestingly, this is also the same time point that corresponded with an increase in CD8 T cell activation (data not shown) and the onset of tumour regression in mice receiving > 2ng/g/mouse DTX, suggesting that temporary removal of Treg can affect the developing tumour.

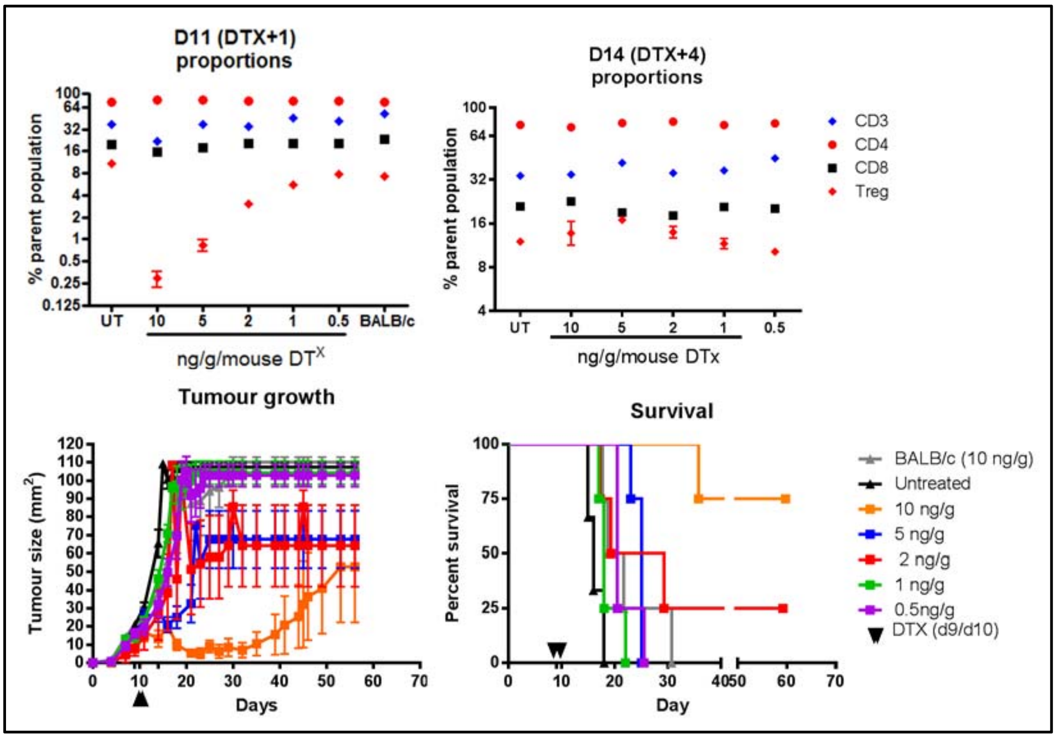

Figure 3: DTX mediated depletion of CD4+ FoxP3+ regulatory T cells. FoxP3.dtr mice were treated with DTX i.p. at indicated doses on days 9 and 10 (q1dx2) and the proportion of lymphocyte subsets in the peripheral bloods was assessed by 6-colour flow cytometry one day after DTX administration (DTX+1). No depletion of Treg was observed in untreated controls (UT) or normal mice treated with 10 ng/g/mouse DTX (BALB/c). Treg depletion occurred in DTX treated FoxP3.dtr mice in a dose dependent manner. No adverse side effects were observed in FoxP3.dtr at these levels of DTX.

These data demonstrated the usefulness of the FoxP3.dtr mice for addressing the influence of Treg during tumour development and therefore we continued to use these mice to address the questions outlined in Aims 1a and 1b. Unfortunately, we had some delays in initially establishing a breeding colony of FoxP3.dtr mice at our animal facility with only limited numbers of mice being produced in each litter. Breeding numbers have been increased and we are coming into a position where mice can be ordered in the quantities needed to perform the experiments as detailed in Aims 1a and 1b.

Shown in Figure 4 below are preliminary data from an ongoing experiment in which AB1-HA tumour bearing mice have been left untreated or treated with gemcitabine, with or without DTX mediated Treg depletion.

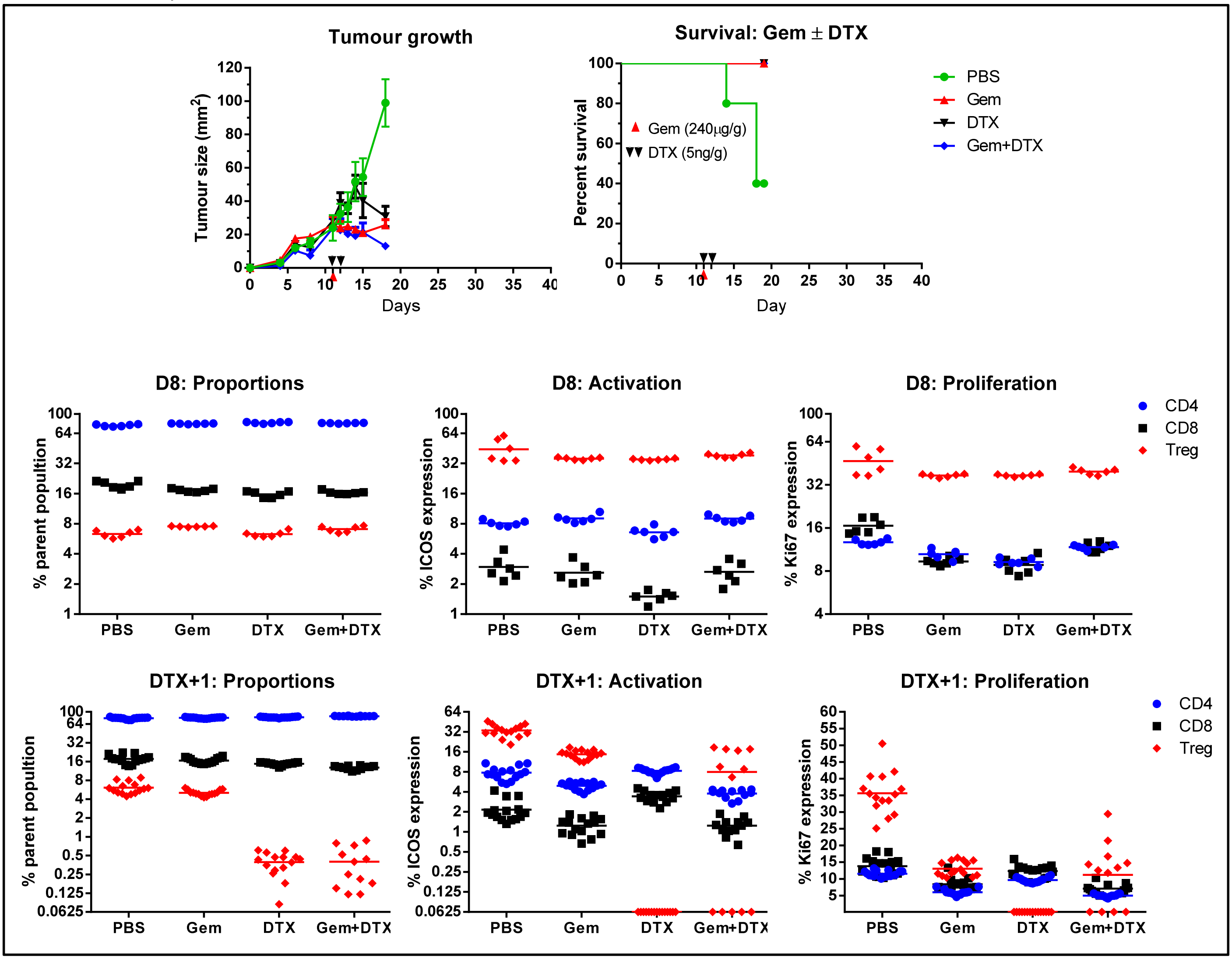

Figure 4: Combination of gemcitabine and Treg depletion on tumour development. Mice were inoculated with tumour s/c on day 0 and treated with 5 ng/g/mouse DTX i.p. (q1dx2) on days 10 and 11. Gemcitabine (240 µg/g/mouse) was administered i.p. on day 10. (A-B) Tumour growth and survival of AB1-HA bearing FoxP3.dtr mice. FACS analysis of CD4 (blue circle), CD8 (Black square) and FoxP3+ CD4+ Treg (red diamond) lymphocyte subset proportions, activation (ICOS+) and proliferation (Ki67+) stutus; (C-E): baseline readings day 9 and (F-H): one day after DTX administration (DTX+1 = day 13).

There was no significant difference in tumour size or T cell phenotype proportion, activation or proliferation status between groups at baseline (i.e. prior to DTX and gemcitabine administration). DTX treatment resulted in a significant decrease in the overall percentage of Treg (as a proportion of total CD4 T cells), with less than 1% of Treg remaining. Gemcitabine treatment did not seem to affect the proportion of CD4, CD8 or Treg lymphocyte populations at this early time point, however as expected, a significant reduction in the proliferation status of all lymphocyte subsets was observed at DTX+1. Mean tumour size in treated groups remain low and show signs of regression in comparison to the untreated control group, in which 60% (3/5 mice) have already been euthanized due to tumours reaching maximum allowable size. Based on our earlier experiments with FoxP3.dtr mice, we expect the onset of tumour regression to correlate with an increase in CD8 T cell activation in the DTX only treated group. However, as the DTX+7 data was not available at the time this report was written this remains to be confirmed. Likewise, tumour regression observed in the Gem treated groups due to the cytotoxic effect of this drug cannot be ruled out and any immunological effect remains to be confirmed at later time points. This experiment is ongoing.

Future Work for Aims 1a and 1b.

Initial assessment of the effect of gemcitabine treatment in the presence of absence of Treg using the FoxP3.dtr mice has some promising results and we are in the process of completing these experiments as more of the FoxP3.dtr mice become available from our increased breeding stocks. All experiments and reagents are at hand and we anticipate that by late November/early December 2013 our FoxP3.dtr mouse stocks will be at a level to maintain the remaining experiments planned for Aim 1b: Optimising the timing and dose of gem+Treg depletion. We plan to have these experiments completed by early 2014.

Aim 1c: Assess optimised treatment parameters in the clinically relevant MexTAg mouse model.

The efficacy of combination gemcitabine chemotherapy and low dose cyclophosphamide (immunotherapy) was tested in the asbestos-induced mesothelioma mouse model. Dosage was based on our previous experiments in which gemcitabine significantly prolonged survival of asbestos-induced mesothelioma, and cyclophosphamide (50 µg daily) significantly reduced the numbers of Tregs (Robinson, unpublished data).

At this optimised dosage gemcitabine significantly extends survival to about the same extent as found in human mesothelioma patients (allowing for species life time expectancy). As expected, cyclophosphamide as a single treatment does not improve survival. In combination, these treatments significantly prolong survival compared to the gemcitabine single treatment group (Figure 5).

Figure 5. Survival of asbestos-induced mesothelioma in mice treated with chemo and immunotherapy. Disease was induced in MexTAg mice by installation of asbestos, 16 weeks later gemcitabine and low dose cyclophosphamide treatments were commenced. Mice were monitored for mesothelioma development and euthanized at the ethics approved endpoint. **p=0.0093, ***p=0.0005 compared to untreated control (Ctrl) group.

Analysis of T cell subsets showed that Treg cells are depleted in the presence of low dose cyclophosphamide (Figure 6).

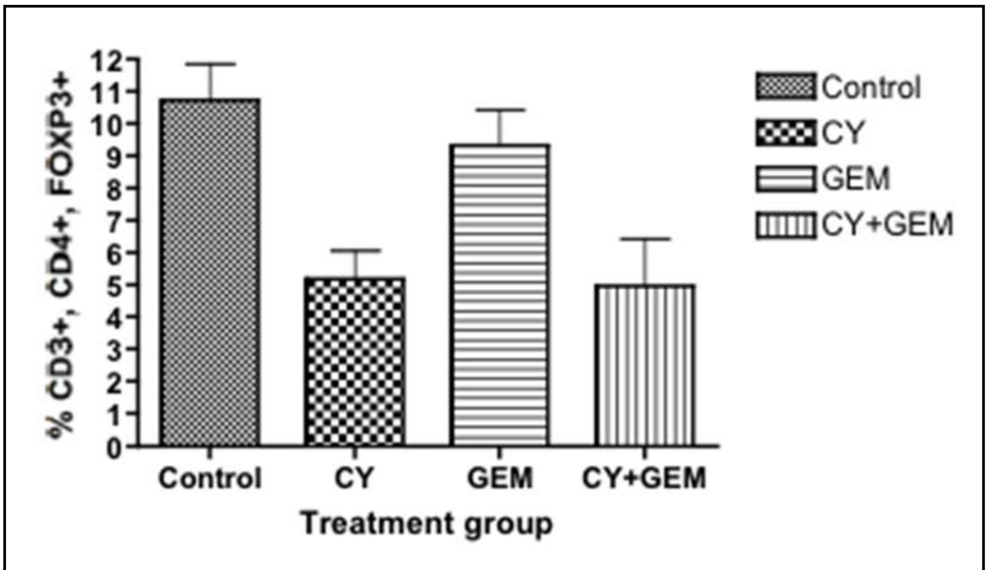

Figure 6: Cyclophosphamide significantly reduces regulatory T cells. Treg cells were identified as staining positive for antibodies to CD3, CD4 and FOXP3 using flow-cytometry. The percentage of Tregs out of total lymphocyte population, 6 weeks after start of treatment, confirmed that Tregs numbers had been reduced compared to untreated controls. P=0.0084.

The experiment will be repeated according to the experimental plan and other treatment combinations will be introduced when information regarding dosage and scheduling has been confirmed in aim 1b.

Aim 2a: To assess the role the adaptive immune system plays during early stages of mesothelial cell transformation.

To assess the role the adaptive immune system plays during early stages of mesothelioma development, immune competent (MexTAg) and immune deficient (MexTAg Rag-/-) mice were exposed to asbestos and monitored for disease development. While we had hypothesised that the immune system played a role in controlling tumour development, to our surprise the results from this experiment clearly indicated the opposite; after mesothelioma induction using asbestos fibres, disease induction was slower in mice lacking B and T cells (p=0.0002, Figure 7).

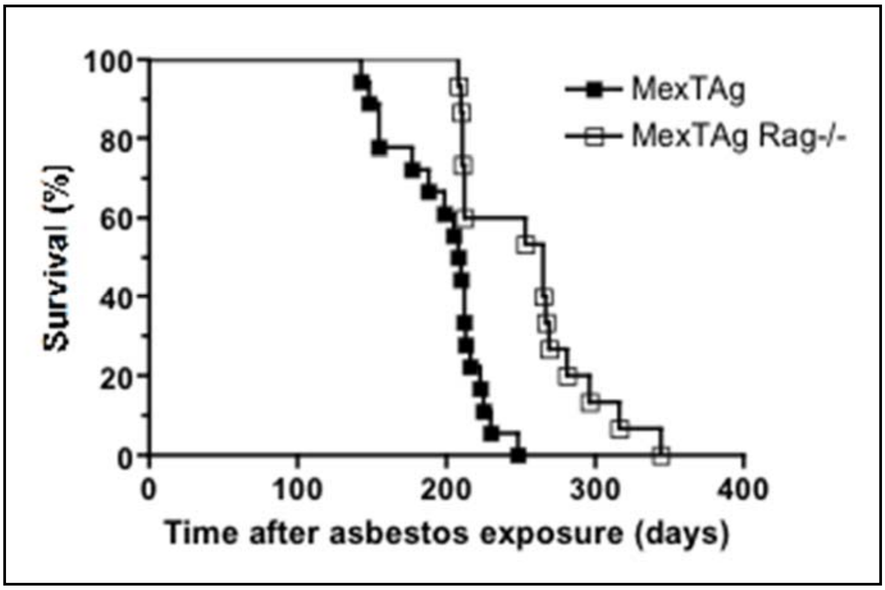

Figure 7. Survival of asbestos induced mesothelioma in transgenic mice with (MexTAg) and without (MexTAgRAG-/-) a functional immune system.

To confirm development of mesothelioma in MexTAgRAG-/- mice, ascites fluid was collected and cell lines were generated. The cell lines are tested for malignancy by injection into the flank of syngeneic mice and assessed for tumour growth. This part of the project is ongoing, however, so far 2/2 ascites lines have formed mesotheliomas. A repeat mesothelioma induction experiment is underway to confirm these interesting results.

Aim 2b Identify the key components of the anti-tumour adaptive immune response.

This study has not begun yet because we are currently accumulating sufficient numbers of mice to set the experiment up and determining the optimum time to start the adoptive transfers from Aim 2a. Experiments are planned to begin in early 2014.

Key Research Accomplishments.

Aims 1a, 1b and 1c:

- Treg inhibitors P60, CCR4 antagonist AF399 and very low dose cyclophosphamide are not effective in our AB-HA mouse model.
- Regulatory T cells can be specifically targeted and efficiently depleted in a dose dependent manner following Diphtheria toxin treatment of FoxP3.dtr.cslr transgenic mice.
- Despite Treg depletion being transient, targeted removal of Treg results in increased CD8 T cell activation which correlates with tumour regression.
- Cisplatin and Pemetrexed have do not affect AB1-HA tumour development in BALB/c mice.
- Gemcitabine (Gem) and cyclophosphamide (high dose) significantly delays AB1-HA tumour development, sometimes resulting in complete tumour regression in BALB/c mice.
- Combination of Gem and CY in MexTAg mice significantly prolongs survival relative to either treatment alone and untreated controls.

Aim 2:

- Asbestos induced mesothelioma developed is significantly slower in immune compromised MexTAg Rag-/- mice compared to immune competent MexTAg mice.

Reportable outcomes:

Manuscripts and Presentations: At least one manuscript will be prepared once the outcomes of Aim2 are known and we plan to present this work in either oral or poster presentation at the biannual International Mesothelioma Interest Group (IMIG) in Cape Town in October 2014.

Cell lines: A number of cell lines have been established from ascites taken from asbestos exposed MexTAg mice associated with this projected. Once confirmed as mesothelioma, these cell lines will be stored in our research facility for future research purposes.

Funding Applications: Data generated from this project has not been used in any additional funding applications as yet. Although once complete we envisage that this work will be used to support a number of local, national and international funding applications.

Conclusions

Despite some initial setbacks in initial testing of Treg inhibitors associated with Aims 1a and b we have made significant progress towards achieving the goals associated with these aims. While initial progress with the FoxP3.dtr mice had been slow due to limited mouse numbers, we are confident that with the increased breeding capacity we will have all the remaining experiments completed by early 2014.

Many of the experiments associated with Aims 1c through 2c are remain ongoing due to the long term nature of experiments associated with our MexTAg models. While some of these experiments have produced some very interesting results, we feel that it is too early to comment on their overall significance at this point and will wait until the remaining experiments can confirm their results.

References

Nil

Appendices

Nil